COMPOSITIONS AND METHODS FOR THE TREATMENT OF VIRAL HEPATITIS
ZUSAMMENSETZUNGEN UND METHODEN ZUR BEHANDLUNG VIRALER HEPATITIS
COMPOSITIONS ET PROCÉDÉS POUR LE TRAITEMENT DE L'HÉPATITE VIRALE

Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC MT NL PL PT RO SE SI SK TR

Priority: 01.12.2006 HU 0600894

Date of publication of application: 23.09.2009 Bulletin 2009/39

Proprietor: HepC Terápia Kereskedelmi és Szolgáltató
Zártkörűen Működő Részvénytársaság
H-1012 Budapest (HU)

Inventors:
• BAKÁCS, Tibor
1012 Budapest (HU)
• KÖVESDI, Imre
Rockville, Maryland 20855-1033 (US)
• PALYA, Vilmos
1223 Budapest (HU)

Representative: Lengyel, Zsolt
Danubia
Patent & Law Office LLC
Bajcsy-Zsilinszky út 16
1051 Budapest (HU)

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WO-A-86/00529

• BAKACS T ET AL: "Examination of the value of treatment of decompensated viral hepatitis patients by intentionally coinfecting them with an apathogenic IBDV and using the lessons learnt to seriously consider treating patients infected with HIV using the apathogenic hepatitis G virus” VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 23, no. 1, 15 November 2004 (2004-11-15), pages 3-13, XP004618116 ISSN: 0264-410X cited in the application

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This invention pertains to a novel attenuated, viral replication inhibitory strain of Infectious Bursal Disease Virus (IBDV) designated V903/78 suitable for harmless propagation in human liver cells, and compositions comprising the same for modulating viral liver disease in a mammal, as well as recombinant IBDV vectors comprising the said IBDV strain and uses thereof.

When hepatitis B, C viruses (HBV and HCV respectively) persist in a chronic carrier state, they serve as a reservoir for infection and give rise to chronic hepatitis and cirrhosis that usually progress to hepatocellular carcinoma, one of the most common malignant tumors with an extremely poor prognosis. Currently approved treatments for chronic hepatitis B - interferon, lamivudin and adefovir - are limited by low rates of sustained response, side effects, or drug resistance. Managing patients with HCV infection consists primarily of antiviral treatment, currently with peginterferon and ribavirin.

IBDV has a worldwide distribution and can cause considerable damage to the poultry industry. IBDV is the causative agent of acute or immunosuppressive disease in chickens. Some zoonotic diseases are of continuing concern, however, IBDV is not known to be a hazard in transmitting to other species despite its worldwide distribution in the domestic fowl (Kibenge et al., 1988); (Pedersden et al., 1990). IBDV is known to exert an inhibitory effect on the replication of hepatitis A virus in monkeys (Csatary et al., 1984). A bursa virus superinfection strategy has been tested for the treatment of acute B and C viral hepatitis in patients (Csatary et al., 1998). The use of IBDV as therapeutic agent in patients suffering from chronic hepatitis infections has also been reported (Bakacs and Mehrishi, 2002); (Csatary et al., 1999).

Given the hurdles associated with the development of anti HCV and HBV agents, there remains a need for improved therapy for both acute and chronic viral liver infections. This can be achieved by the use of IBDV vectors that have great flexibility in their construction and use, and can provide greater success in the treatment of viral liver diseases. An IBDV vector that can replicate in human liver cells without causing detrimental effects to them would be ideal. The present invention provides such vectors, and the use of such vectors.

The present invention relates to an attenuated clonal, viral replication inhibitory strain of Infectious Bursal Disease Virus (IBDV) comprising the RNA nucleotide sequence of the complete virus designated IBDV V903/78 as depicted in SEQ. ID. NO: 1 and 2 which can be grown in the HepG2 human liver cell line without causing detrimental effects to the cells. The invention also provides a recombinant attenuated IBDV vector comprising the nucleotide sequence of the said IBDV strain and further comprising sequence elements for the inclusion and/or expression of exogenous nucleic acid sequences. These exogenous nucleic acid sequences can enhance the therapeutic effects of the said IBDV vector. In one preferred embodiment of the invention the exogenous nucleic acid sequence encodes a cytokine. The preferred cytokine encoded belongs to the family of interferons.

The present invention also provides pharmaceutical compositions comprising, said viral replication inhibitory strain of IBDV or said IBDV vector and a suitable carrier or excipient. In one preferred embodiment the pharmaceutical composition comprises $10^{5.0} - 10^{8.0}$ 50% tissue culture infecting dose (TCID$_{50}$) per unit dose or, more advantageously, $10^{6.3} - 10^{7.0}$ TCID$_{50}$ per unit dose.

The present invention provides a viral replication inhibitory strain of IBDV or an IBDV vector or a composition for use in therapy and in one preferred embodiment for use in the treatment or prevention of viral infections, preferably hepatitis.

Detailed Description of the Invention

By the expression "viral replication inhibitory strain" we mean an IBDV strain that efficiently limits the replication of a different virus, preferably a hepatitis B or hepatitis C virus, present in the same animal.

Generally an "attenuated strain" is a strain that has been altered to exhibit diminished virulence. In the case of the IBDV strain of the invention this means that it does not induce disease in specific pathogen free (SPF) chickens following artificial infection.
A "functionally equivalent" derivative of the IBDV strain of the invention, as used herein, is a derivative which retains its capability of inhibiting replication of a co-existing virus (preferably a hepatitis B or hepatitis C virus) and of being propagated in the HepG2 (ATCC Number HB-8065) human liver cell line without causing detrimental effects to the cells.

The present invention provides an apathogenic, attenuated avian virus, IBDV, as antiviral agent. IBDV belongs to the genus Avibirnavirus and is a member of the family Birnaviridae. The Birnaviridae family is known to have very narrow species limits. It was therefore quite unexpected that V903/78 was able to replicate in the human hepatoma cell line, which is separated by several hundred million years in evolution from the birds. Furthermore, it is very important to note that the IBDV replication was not associated with cytopathic effects in the HepG2 cells. Therefore, this human cell line can be used for model studies of viral interference between V903/78 virus and other viruses. IBDV remains infective at pH 2.0 and can be produced in primary chicken cell or VERO cell cultures that have been certified for other vaccines.

The VERO epithelial cell line was established by Y. Yasumura and Y. Kawakita in 1962 at the Chiba University in Chiba, Japan. The tissue from which the line was derived was obtained from the kidney of a healthy adult African green monkey. VERO cells are commercially available and are widely used in transfections and vaccine production (ATCC Number CCL-81).

**IBDV Genome**

IBDV is a non-enveloped icosahedral virus particle of 60 nm in diameter, which contains two genome segments of double-stranded RNA (see fig. 2). Genome segment A determines the bursa tropism of IBDV, whereas segment B is involved in the efficiency of viral replication. Significantly, the interaction of the two segments, the polymerase (segment B) with the structural protein VP3 (segment A) are necessary for efficient virus formation and replication (Zierenberg et al., 2004). The larger segment A encodes a 110 kDa precursor protein in a single large open reading frame (ORF), which is cleaved by autoproteolysis to yield mature VP2, VP3 and VP4 proteins. VP2 is the major host-protective immunogen of IBDV. The smaller segment B encodes VP1, a 97 kDa protein having RNA-dependent RNA polymerase activity. IBDV infects the precursors of antibody-producing B cells in the bursa of fabriceus, which can cause immune-suppression and mortality in young chickens. Studies have shown that virulent strains of IBDV lose their virulence potential after serial passage in non-B lymphoid chicken cells. Comparison of the deduced amino acid sequences of the virulent and attenuated strains shows specific amino acid substitution within the hypervariable region of the VP2 protein.

**Characterization of IBDV strain V903/78**

Strain V903/78 of IBDV, the antiviral agent according to the present invention for the treatment of chronic viral hepatitis B and C infections was obtained from domestic poultry in Hungary in 1978. The virus strain was isolated from the bursal tissues of a 3-week-old healthy broiler chicken by inoculation of 11-day-old embryonated specific pathogen free (SPF) eggs. The virus grown in the embryonated eggs was adapted to VERO cell culture. After 16 passages in the cell line, the strain was plaque-purified once and a stock of vims designated as V903/78 passage 19 was produced for genomic characterization. Said strain does not induce disease in SPF chickens following artificial infection, therefore this strain can be considered attenuated. This statement is supported by sequence analysis data of the VP2 gene of the V903/78 strain, which shows the closest relationship with other tissue adapted vaccine strains (see the phylogenetic free on fig. 3).

The V903/78 virus stock was three times further plaque-purified and grown in VERO cell culture. This viral stock was sequenced again and was used to create the Master Seed Virus (MSV) stock. The entire nucleotide sequence of the complete virus designated IBDV V903/78 (see Figs. 1.A and 1.B) is depicted in SEQ. ID. NO: 1 (segment A) and SEQ. ID. NO: 2 (segment B).

**Recombinant Vector Constructs**

The present invention also provides recombinant IBDV vectors that allow for the inclusion of exogenous nucleic acid sequences. The nucleic acid sequences are operably linked to regulatory sequences necessary for expression of protein coding or non-coding sequences that enhance the therapeutic effects of the IBDV vector.
IFN-alpha, IFN-beta and TNF-alpha induction by IBDV strain V903/78 in mice liver

(numbered by Bayliss et al., 1990), which encompasses the hypervariable region of the VP2 gene. V903/78 is verified by PCR. A nested PCR method is used to amplify a 414 bp product spanning from 750 to 1163 nt inoculation. IBDV virus titre determination of samples is done by standard titration method. The presence of IBDV strain supernatants containing lysed cells (after three freeze-thaw cycle) are taken at 24 hour intervals up to 120 hour post-10^6.3 TCID50/0.1 ml). Next day all mice are sacrificed and samples are collected from feces. The feces are diluted into media for 5 days at 37°C in a 5% CO2 atmosphere, cultures are microscopically monitored every day for cytopathic effect (CPE) at 10, 20 and 40x magnification, and photographs are taken at the 5th day of culture. Cell free culture supernatants and virus is grown on confluent adherent cell cultures of African green monkey VERO cell line. VERO cells are incubated for 5 days at 37°C in a 5% CO2 atmosphere, cultures are microscopically monitored every day for cytopathic effect (CPE) at 10, 20 and 40x magnification, and photographs are taken at the 5th day of culture. Cell free culture supernatants and supernatants containing lysed cells (after three freeze-thaw cycle) are taken at 24 hour intervals up to 120 hour post-inoculation. IBDV virus titre determination of samples is done by standard titration method. The presence of IBDV strain V903/78 is verified by PCR. A nested PCR method is used to amplify a 414 bp product spanning from 750 to 1163 nt (numbered by Bayliss et al., 1990), which encompasses the hypervariable region of the VP2 gene. **Stability of IBDV strain V903/78 after in vivo oral delivery in mice gut**

Five 6-week-old Balb/c mice are inoculated orally with 0.1 ml of 10^6.3 IBDV strain V903/78 (virus titre: 10^6.3 TCID50/0.1 ml). Next day all mice are sacrificed and samples are collected from feces. The feces are diluted into media and virus is grown on confluent adherent cell cultures of African green monkey VERO cell line. VERO cells are incubated for 5 days at 37°C in a 5% CO2 atmosphere, cultures are microscopically monitored every day for cytopathic effect (CPE) at 10, 20 and 40x magnification, and photographs are taken at the 5th day of culture. Cell free culture supernatants and supernatants containing lysed cells (after three freeze-thaw cycle) are taken at 24 hour intervals up to 120 hour post-inoculation. IBDV virus titre determination of samples is done by standard titration method. The presence of IBDV strain V903/78 is verified by PCR. A nested PCR method is used to amplify a 414 bp product spanning from 750 to 1163 nt (numbered by Bayliss et al., 1990), which encompasses the hypervariable region of the VP2 gene.

**Stability of IBDV strain V903/78 after in vivo oral delivery in mice gut**

IFN-alpha, IFN-beta and TNF-alpha induction by IBDV strain V903/78 in HepG2 cell line

Induction of cytokines such as IFN-alpha, IFN-beta and TNF-alpha is investigated in human hepatocytes by IBDV strain V903/78 infection. HepG2 cells are infected with V903/78 virus at a multiplicity of infection (MOI) of approximately 1 to 10 plaque forming unit (pfu). Between 1 and 10 days post-infection, cytokine induction is measured from the supernatant media by ELISA measurements. The ELISA measurements performed for IFN-alpha, IFN-beta and TNF-alpha with the use of commercially available ELISA kits. The levels of cytokines present in the supernatant of infected HepG2 cells are compared to uninfected HepG2 cells. At 10 days post-infection intracellular RNA is harvested from the cells. The quantities of cytokine mRNAs are determined by RT-PCR with cytokine specific primers, and the levels of specific mRNAs are compared to uninfected HepG2 cells.

**Stability of IBDV strain V903/78 after in vivo oral delivery in mice gut**

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**Stability of IBDV strain V903/78 after in vivo oral delivery in mice gut**

IFN-alpha, IFN-beta and TNF-alpha induction by IBDV strain V903/78 in mouse liver

On day 1, five 6-week-old Balb/c mice are inoculated orally with 0.1 ml of 10^6.3 IBDV strain V903/78 (virus titre: 10^6.3 TCID50/0.1 ml). On day 2 and day 3 the mice are repeatedly infected orally by the same amounts of IBDV. On day 5 all mice are sacrificed and samples are collected into liquid nitrogen for viral culture and histological studies from lymph nodes, spleen, liver, lung, kidneys, intestine, Peyer patches, thymus. Liver and intestine samples are also collected for ELISA measurements. The tissues are ground and ELISA measurements performed on the tissue lysates for IFN-alpha, IFN-beta and TNF-alpha with the use of commercially available ELISA kits. The levels of cytokines in infected animal
tissues are compared to uninfected animal tissue.

Viral interference between V903/78 virus and DHBV (Duck Hepatitis Virus) in vitro in HepG2 cells

[0027] To demonstrate that IBDV inhibits HBV/HCV replication directly by viral interference, superinfection is carried out such that HepG2 cells are transfected with an infectious DHBV plasmid and then superinfected with V903/78 virus. Superinfection is carried out by transfecting HepG2 cells with closed circular DHBV DNA as described by Galle et al., J Virol (1988) 62:1736-40. Circular DNA is prepared from plasmid pD16, which carries a full-length DHBV type 16 (DHBV-16) genome in the EcoRI site of pUC13. Full-length DHBV DNA is excised with EcoRI, circularized with T4 DNA ligase, and introduced into cells by calcium phosphate transfection. Sub-confluent HepG2 cells are passaged at a 1:5 dilution; 20 h later the medium is changed, and another 2 h later 1 ml of transfection cocktail (10 μg of DNA, 438 μl of Tris [pH 7.6], 62 μl of 2 M CaCl₂, 500 ml of HEPES-buffered saline [280 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid]) is added per 75 cm² dish. After 12 h, dimethyl sulfoxide is added to a final concentration of 10%, and the cells are incubated at 37°C for 20 min. The medium is changed afterwards. The cells are then superinfected with V903/78 virus 1, 2, 5 days later at an MOI of approximately 1 to 100 pfu. Between 6 and 10 days post-infection, intracellular viral DNA and RNA respectively are harvested, amplified by PCR and RT-PCR respectively, and analyzed for the presence of DHBV and V903/78 virus genomes to determine viral interference between the two viruses.

Viral interference between V903/78 virus and its derivatives and DHBV in vivo in the duck model of HBV

[0028] The avian duck hepatitis B virus model system is used for studying the clinically observed efficacy of IBDV superinfection therapy. Newborn Pekin ducklings, either congenitally infected with DHBV type 16 or uninfected, are obtained from the University of Alberta, Edmonton, Alberta, Canada. Wild-type DHBV is obtained from the serum of congenitally infected ducks. Viral titers are quantitated before and after IBDV treatment by dot blotting with plasmid standards and are expressed as viral genome equivalents (VGE). The technical details are described in the paper of Walters et al. (Walters et al., 2004).

[0029] Five newborn ducklings congenitally infected with DHBV in each of the 6 groups are inoculated orally with 0.1 ml of 10⁻⁶.³ IBDV strain V903/78; 903/78INFb, 903/78INFa, 903/78INFgP and 903/78INFgD viruses (virus titre: 10⁻⁶.³ TCID₅₀/0.1 ml). One group that is not superinfected with IBDV is used as control. Two DHBV uninfected ducklings are used as negative controls. IBDV treatment is repeated every 3 days and the health and weight of the animals are monitored. Reduction of the DHBV virus titers are monitored in the serum every ten days by PCR methodology.

[0030] Extracellular viral DNA is extracted from serum for analysis. Twenty microliters of serum is added to 80 μl of 50 mM Tris-HCl (pH 8)-150 mM NaCl- 10 mM EDTA-0.1% sodium dodecyl sulfate (SDS)-800 μg of proteinase K/ml and incubated at 42°C for a minimum of 4 h. The sample is extracted with an equal volume of phenol-chloroform. DNA is precipitated by adding a 0.1 volume of 3 M sodium acetate, 10 μg of yeast tRNA, and 2 volumes of 95% ethanol. The DNA is resuspended in 20 μl of water. Ten microliters is used for a subsequent PCR. The extracted viral DNA is amplified by PCR with Taq polymerase (Gibco BRL) according to the manufacturer’s specifications, 1.5 mM MgCl₂, and the following primers at 0.25 μM: 5′-CTCAAGAGATTCCTCAGCC-3′ (SEQ. ID. NO: 3) and 5′-GTCATACCATTCTCCTACT-3′ (SEQ. ID. NO: 4). Cycling conditions are as follows: 95°C for 4 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and 72°C for 7 min. The PCR products are separated on 1.3% agarose gels and visualized with ethidium bromide to observe the 906 bp DHBV specific products.

[0031] On day 30 post IBDV infection, all ducks are sacrificed and samples are collected into liquid nitrogen for viral culture, histological studies, PCR and ELISA for cytokines (IFN-alpha, IFN-beta, IFN-gamma and TNF-alpha) from lymph nodes, spleen, liver, lung, kidneys, intestine, Peyer patches, thymus. Reduction of the DHBV virus titers are also evaluated by DNA dot blot and quantitative PCR methodologies.

Single or multiple deficient vectors and complementing cell lines

[0032] For various applications the use of single or multiple deficient vectors may be highly advantageous. A deficient IBDV vector could be complemented by a cell line that expresses the missing function of the vector. An example of this would be an IBDV vector missing or having a non-functional polymerase. The smaller segment B of IBDV encodes VP1, a 97 kDa protein having RNA-dependent RNA polymerase activity. If this segment is expressed in a production cell line (eg. VERO cells) that cell line allows the manufacture of an RNA polymerase deficient IBDV vector by complementarity (see e.g. US 7,195,896).
BRIEF DESCRIPTION OF THE DRAWINGS

[0033] The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the drawings.

Figure 1A and 1B depicts the complete nucleotide sequences of Segment A and Segment B, respectively, of IBDV strain V903/78 of the invention.

Figure 2. The genome of IBDV consists of two segments of double-stranded RNA. The larger segment A encodes a 110 kDa precursor protein in a single large open reading frame (ORF), which is cleaved by autoproteolysis to yield mature VP2, VP3 and VP4 proteins. VP2 is the major host-protective immunogen of IBDV. The smaller segment B encodes VP1, a 97 kDa protein having RNA-dependent RNA polymerase activity.

Figure 3. The phylogenetic tree shows that the strain V903/78 has closest relationship with other tissue adapted vaccine strains.

Figure 4. Replication of an attenuated IBDV strain 903/78 in the human hepatoma cell line. A - HepG2 cell control at 40x magnification. B - IBDV infected HepG2 cells at 40x magnification.

Figure 5. Schematic diagram of cDNA construct and primers used for the construction of pC903-INFb plasmid.

Figure 6. Schematic diagram of cDNA construct and primers used for the construction of pC903-INFgP (proximal) and pC903-INFgD (distal) plasmids.

Figure 7. Schematic diagram of the pC903-AflII plasmid and the cDNA construct and primers used for the construction of the pC903-INFa plasmid.

EXAMPLES

[0034] The following examples further illustrate the present invention but should not be construed as in any way limiting its scope.

Example 1

IBDV strain V903/78 is grown in the HepG2 human liver cell line without causing detrimental effects to the cells.

[0035] Virus replication was measured in half confluent adherent cell culture of HepG2 cells. Uninfected cells were used for controls (Figure 4A). The TCID_{50} titers of IBDV V903/78 seed virus produced on HepG2 cells were 10^{5.0}/0.1 ml on HepG2 and 10^{6.0}/0.1 ml on chicken embryo fibroblast (CEF) cells. 11.6 x 10^5 HepG2 cells were seeded into a 25 cm^2 tissue culture flask, which was inoculated by 1 ml of 500x diluted seed virus. HepG2 cells were incubated for 5 days at 37°C in a 5% CO_2 atmosphere, cultures were microscopically monitored every day (at 10, 20 and 40x magnification), and photographs were taken at the 5th day of culture (Figure 4B). Following 5 days of culture, supernatants were harvested and titrated on CEF cells. The titers of harvest taken from culture inoculated at half confluence were 10^{4.7}/0.1 ml on HepG2 and 10^{7.4}/0.1 ml on CEF cells.

[0036] The replication kinetics of the attenuated IBDV strain V903/78 was compared in the African green monkey VERO cell line and in the human hepatoma HepG2 cell line. Virus replication was measured by carrying out inoculation in both suspension and in adherent cell cultures, respectively. For testing in suspension cultures, 25 cm^2 tissue culture flasks were seeded with 10^{6.20} (1.6 x 10^6) VERO cells or with 10^{6.13} (1.4 x 10^6) HepG2 cells, and inoculated at a MOI of approximately 1 at the time of seeding with the IBDV strain V903/78 (Table 1). For testing in confluent adherent cell cultures, the cell counts in 25 cm^2 tissue culture flasks were 10^{6.46} (2.9 x 10^6) for VERO cultures and 10^{6.39} (2.5 x 10^6) for HepG2 cultures at the time of inoculation (infection) with IBDV strain V903/78, respectively (Table 1). VERO and HepG2 cells were incubated for 5 days at 37°C in a 5% CO2 atmosphere; cultures were microscopically monitored every day (at 10, 20 and 40x magnification). Cell free culture supernatants and supernatants containing lysed cells (after a freeze-thawed cycle) were taken at 24 hr intervals up to 120 hr post-inoculation from tissue culture flasks of each type of cell culture. Virus titre determination of samples was done by standard titration method on CEF cell cultures. The results of IBDV V903/78 virus replication in HepG2 and VERO cell lines are summarized in Table 2 below. It is very important to note that the IBDV replication was not associated with cytopathic effects in the HepG2 cells (Figure 4 B).

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Cell line</th>
<th>Cell count at infection (x 10^6 cell/flask)</th>
<th>Titre of virus at inoculum (log10)</th>
<th>Virus/cell ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherent</td>
<td>HepG2</td>
<td>2.47</td>
<td>6.39</td>
<td>0.94</td>
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</table>

Table 1. Virus titre (log10) and virus/cell ratio in different cell lines at infection time.
Example 2

The construction of a recombinant IBDV vector incorporating a third genomic segment expressing a cytokine

Methods for generating birnavirus from synthetic RNA transcripts is described in Mundt, E., and Vakharia, V. N. (1996). Proc Natl Acad Sci U S A 93, 11131-11136, U.S. Pat. No. 5,871,744. Full-length molecular cDNA clones of the A (pA903) and B (pB903) segments of the IBDV strain V903/78 are constructed according to the methods in Mundt, E., and Vakharia, V. N. (1996). Proc Natl Acad Sci USA 93, 11131-11136. A third genomic segment, denoted as segment C (pC903-INFb) is constructed to express human INF-beta. The recipient plasmid pB903 is comprised of the B segment that is the 5' and 3' non-coding regions and of the VP1 protein coding region.

First, the INF-beta sequence is amplified by PCR with the INF-beta specific primers:

aBb1: 5'-GGTTCCCATCATGGCTGTTACTGGGATGCTCTTCGACCTC-3' (SEQ. ID. NO: 5)
sBb2: 5'-CCTCTTCTTGATGATTCTGCCACCATGTTAATTCTCTCGGAAACG-3' (SEQ. ID. NO: 6).

The human INF-beta plasmid from Geneservice Ltd. (Clone: MGC:96956; GenBank number: NM_002176) is used as a template for this reaction.

Then the IBDV 5' and 3' sequences are amplified using the pB903 plasmid as the template and the two sets of outside primers:

B5'-903: 5'-AGAGAATTCTAATACGACTCACTATAGGATACGATGGGTCTGAC-3' (SEQ. ID. NO: 7)
aBb2: 5'-CGTTTCCGAGAGAATTAACATGGTGGCAGAATCATCAAGAAGG-3' (SEQ. ID. NO: 8)
and
B3'-903: 5'-CGATCTGCTGCAGGGGGCCCCCGCAGGCGAAGG-3' (SEQ. ID. NO: 9)
sBb1: 5'-GAGGTCGAAGAGCATCCCAGTAACAGCCATGATGGGAACC-3' (SEQ. ID. NO: 10).

Next aliquots from the two previous reactions are mixed and primers B5'-903 and B3'-903 are used to amplify the complete new sequence (Figure 5). The PCR product is purified and digested with Psi I and EcoRI restriction enzymes and cloned into the Psi I (5' region) and EcoRI (3' region) restriction sites of the pB903 vector resulting in plasmid pC903-INFb. The three plasmids (pA903, pB903 and pC903-INFb) are used as templates for in vitro transcription with T7 RNA polymerase, transfected into VERO cells. Production in the cell supernatant of INF-beta is verified by ELISA and the new virus (903/78INFb) that express INF-beta is selected by standard plaquing.

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Cell line</th>
<th>Cell count at infection (x 10⁶cell/flask)</th>
<th>Titre of virus at inoculum (log10)</th>
<th>Virus/cell ratio</th>
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<tbody>
<tr>
<td>VERO</td>
<td>2.92</td>
<td>6.46</td>
<td>0.88</td>
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<td>Suspension</td>
<td>HepG2</td>
<td>1.37</td>
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<td>VERO</td>
<td>1.60</td>
<td>6.20</td>
<td>0.91</td>
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</tbody>
</table>

Example 2

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Example 3

The construction of a recombinant IBDV vector that incorporates a cytokine coding sequence into an existing genomic segment B

[0042] The full-length molecular cDNA clone of the B (pB903) segments of the IBDV strain V903/78 constructed according to and methods in Mundt, E., and Vakharia, V. N. (1996) is modified by incorporating a new gene (INF-gamma) front of (proximal) or behind (distal) position of the VP1 coding region. The cDNA constructs are made as in Example 2 with primers shown in Figure 6. A Smal restriction site is placed between the two gene-coding sequences (VP1 and INF-gamma) by the use of primers aBg1 and sBg1. The two genes are functionally connected with an internal ribosome entry site (IRES) that is cloned into the Smal site with blunt end ligation. The IRES is derived from the human eIF4G initiation factor according to Wong et al. (2002) Gene Therapy 9:337-344. The INF-gamma sequence is amplified by PCR with the INF-gamma specific primers shown on Figure 6. The human INF-gamma plasmid from Geneservice Ltd. (Clone: MGC: 88243; GenBank number: NM_000619) is used as a template for this reaction.

[0043] The two plasmids (pA903, and pB903-INFgP) and (pA903, and pB903-INFgD) respectively are used as templates for in vitro transcription with T7 RNA polymerase, transfected into VERO cells. Production in the cell supernatant of INF-gamma is verified by ELISA and the new viruses (903/78INFgP) and (903/78INFgD) that express INF-gamma are selected by standard plaquing.

Example 4

The construction of a recombinant IBDV vector that incorporates a cytokine into an existing genomic segment A

[0044] In this example the non-essential protein VP5 is exchanged for INF-alpha2 and an IRES sequence. Full-length molecular cDNA clones of segment A (pA903) of IBDV strain V903/78 are constructed according to Example 2 and methods in Mundt, E., and Vakharia, V. N. (1996). Proc Natl Acad Sci USA 93, 11131-11136. Plasmid p903A was mutated according to Mundt et al. (1997) J. Virology 71:5647-5651. The start codon of VP5 was changed to Arg from Met and an AflII site was created. Similarly another AflII site was created adjacent to the VP2 start codon resulting in plasmid pA903Afl (Figure 7). The INF-alpha sequence is amplified by PCR with the INF-alpha specific primers shown on Figure 7. The human INF-alpha plasmid from Geneservice Ltd. (Clone: MGC: 104046; GenBank number: NM_000605) is used as a template for this reaction. The human eIF4G initiation factor IRES was PCR amplified according to Wong et al. (2002) Gene Therapy 9:337-344., as in Example 3 using primers according to Figure 7. Aliquots from the two previous reactions are mixed and the two outside primers sAal and aAa3 incorporating AflII sites are used to amplify the combined sequence (Figure 7). The PCR product is purified and digested with AflII restriction enzyme and cloned into the AflII cut pA903Afl plasmid, replacing the 5' region of VP5 coding sequences resulting in plasmid pC903-INFa. The two plasmids (pA903-INFa and pB903) are used as templates for in vitro transcription with T7 RNA polymerase, transfected into VERO cells. Production of INF-alpha in the cell supernatant is verified by ELISA, and the new virus (903/78INFa) expressing INF-alpha is selected by standard plaquing.

[0045] All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entirities by reference.

[0046] While this invention has been described with an emphasis upon some preferred embodiments, variations of the preferred embodiments can be used, and it is intended that the invention can be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

REFERENCES


Claims

1. An attenuated clonal, viral replication inhibitory strain of Infectious Bursal Disease Virus (IBDV) comprising the RNA nucleotide sequence of the complete virus designated IBDV V903/78 as depicted in SEQ. ID. NO: 1 and 2, which can be grown in the HepG2 human liver cell line without causing detrimental effects to the cells.
2. An attenuated IBDV strain according to claim 1 which can be produced at least to 10^7.5 /0.1 ml TCID_{50} titre in HepG2 cells.
3. A recombinant attenuated IBDV vector comprising the nucleotide sequence of the IBDV strain as claimed in claims 1 or 2, further comprising exogenous nucleic acid sequences.
4. A recombinant attenuated IBDV vector as claimed in claim 3, further comprising sequence elements for the inclusion and/or expression of exogenous nucleic acid sequences.
5. An IBDV vector as claimed in claim 3 or 4, comprising an exogenous nucleic acid sequence encoding a cytokine.
6. An IBDV vector as claimed in claim 5, wherein the encoded cytokine belongs to the family of interferons.
7. A pharmaceutical composition comprising, a viral replication inhibitory strain of IBDV as claimed in claim 1 or 2 or an IBDV vector as claimed in anyone of claims 3 to 6 and a suitable carrier or excipient.
8. A pharmaceutical composition as claimed in claim 7 comprising 10^{5.0} - 10^{8.0} TCID_{50} per unit dose or, more advantageously, comprising 10^{6.3} - 10^{7.0} TCID_{50} per unit dose.
9. A viral replication inhibitory strain of IBDV as claimed in claim 1 or 2 or an IBDV vector as claimed in anyone of claims 3 to 6 or a composition as claimed in anyone of claims 7 to 8 for use in therapy.
10. A viral replication inhibitory strain of IBDV as claimed in claim 1 or 2 or an IBDV vector as claimed in anyone of claims 3 to 6 or a composition as claimed in anyone of claims 7 to 8 for use in the treatment or prevention of a viral infection.

11. A viral replication inhibitory strain of IBDV as claimed in claim 1 or 2 or an IBDV vector as claimed in anyone of claims 3 to 6 or a composition as claimed in anyone of claims 7 to 8 for use in the treatment or prevention of viral hepatitis.

12. Use of a therapeutically effective amount of live, attenuated IBDV strain as claimed in claim 1 or 2 or an IBDV vector as claimed in anyone of claims 3 to 6 or a pharmaceutical composition as claimed in anyone of claims 7 to 8 for the manufacture of a pharmaceutical composition suitable for the prevention or treatment of viral hepatitis.

13. A system comprising: (i) a deficient IBDV vector as claimed in anyone of claims 3 to 6, and (ii) a cell line that expresses the missing function of said vector.

14. The system according to claim 13, wherein said IBDV vector is deficient in RNA-polymerase expression.

Patentansprüche


21. Attenuierter IBDV-Stamm nach Anspruch 1, welcher wenigstens bis zu einem TCID50-Titer von 10^{7.5} / 0.1 ml in HepG2-Zellen erzeugt werden kann.

22. Rekombinanter, attenuierter IBDV-Vektor umfassend die Nukleotidsequenz des IBDV-Stammes nach Anspruch 1 oder 2, weiterhin umfassend exogene Nukleinsäuresequenzen.

23. Rekombinanter, attenuierter IBDV-Vektor nach Anspruch 3 weiterhin umfassend Sequenzelemente für die Inklusion und/oder Expression von exogenen Nukleinsäuresequenzen.

24. Rekombinanter, attenuierter IBDV-Vektor nach Anspruch 3 oder 4 umfassend eine exogene Nukleinsäuresequenz, welche ein Cytokin kodiert.

25. IBDV-Vektor nach Anspruch 5, wobei das kodierte Cytokin zur Familie der Interferone gehört.

26. Pharmazeutische Zusammensetzung umfassend einen IBDV-Stamm zur Inhibierung der viralen Replikation nach Anspruch 1 oder 2 oder einen IBDV-Vektor nach einem der Ansprüche 3 bis 6 und einen geeigneten Träger oder Hilfsstoff.

27. Pharmazeutische Zusammensetzung nach Anspruch 7 umfassend 10^{5.0} - 10^{6.0} TCID_{50} pro Dosiseinheit oder vor teilhafter umfassend 10^{6.3} - 10^{7.0} TCID_{50} pro Dosiseinheit.

28. IBDV-Stamm zur Inhibierung der viralen Replikation nach Anspruch 1 oder 2 oder IBDV-Vektor nach einem der Ansprüche 3 bis 6 oder Zusammensetzung nach einem der Ansprüche 7 bis 8 zur Verwendung in der Therapie.

29. IBDV-Stamm zur Inhibierung der viralen Replikation nach Anspruch 1 oder 2 oder IBDV-Vektor nach einem der Ansprüche 3 bis 6 oder Zusammensetzung nach einem der Ansprüche 7 bis 8 zur Verwendung in der Behandlung oder Vorbeugung einer viralen Infektion.

30. IBDV-Stamm zur Inhibierung der viralen Replikation nach Anspruch 1 oder 2 oder IBDV-Vektor nach einem der Ansprüche 3 bis 6 oder Zusammensetzung nach einem der Ansprüche 7 bis 8 zur Verwendung in der Behandlung oder Vorbeugung von viraler Hepatitis.

31. Verwendung einer therapeutisch wirksamen Menge eines lebenden, attenuierten IBDV-Stamms nach Anspruch 1 oder 2 oder eines IBDV-Vektors nach einem der Ansprüche 3 bis 6 oder einer Zusammensetzung nach einem der
Ansprüche 7 bis 8 für die Herstellung einer pharmazeutischen Zusammensetzung, welche für die Vorbeugung oder Behandlung von viraler Hepatitis geeignet ist.

13. System umfassend: (i) einen defizienten IBDV-Vektor nach einem der Ansprüche 3 bis 6, und (ii) eine Zelllinie, die die fehlende Funktion des Vektors exprimiert.


**Revendications**

1. Souche clonale atténuée inhibitrice de réplication virale du virus de la bursite infectieuse (IBDV) comprenant la séquence nucléotidique d’ARN du virus complet désigné par IBDV V903/78 comme décrit dans les SEQ ID n° 1 et 2, qui peut croître dans la lignée cellulaire du foie humain HepG2 sans provoquer d’effets préjudiciables pour les cellules.

2. Souche d’IBDV atténuée selon la revendication 1, qui peut être produite au moins au titre de\(10^{7.5}\)/0,1 ml de TCID\(_{50}\) dans les cellules HepG2.

3. Vecteur d’IBDV atténué recombinant comprenant la séquence nucléotidique de la souche d’IBDV selon la revendication 1 ou la revendication 2, comprenant en outre des séquences d’acides nucléiques exogènes.


5. Vecteur d’IBDV selon la revendication 3 ou la revendication 4, comprenant une séquence d’acide nucléique exogène codant pour une cytokine.

6. Vecteur d’IBDV selon la revendication 5, dans lequel la cytokine codée appartient à la famille des interférons.

7. Composition pharmaceutique comprenant une souche d’IBDV inhibitrice de réplication virale selon la revendication 1 ou la revendication 2 ou vecteur d’IBDV selon l’une quelconque des revendications 3 à 6 et un véhicule ou un excipient approprié.

8. Composition pharmaceutique selon la revendication 7, comprenant \(10^{5.0} \text{ à } 10^{6.0}\) de TCID\(_{50}\) par unité de dose et, plus avantageusement, comprenant \(10^{6.3} \text{ à } 10^{7.0}\) de TCID\(_{50}\) par unité de dose.

9. Souche d’IBDV inhibitrice de réplication virale selon la revendication 1 ou la revendication 2 ou vecteur d’IBDV selon l’une quelconque des revendications 3 à 6 ou composition selon l’une quelconque des revendications 7 à 8 pour usage en thérapie.

10. Souche d’IBDV inhibitrice de réplication virale selon la revendication 1 ou la revendication 2 ou vecteur d’IBDV selon l’une quelconque des revendications 3 à 6 ou composition selon l’une quelconque des revendications 7 à 8 pour usage dans le traitement ou la prévention d’une infection virale.

11. Souche d’IBDV inhibitrice de réplication virale selon la revendication 1 ou la revendication 2 ou vecteur d’IBDV selon l’une quelconque des revendications 3 à 6 ou composition selon l’une quelconque des revendications 7 à 8 pour usage dans le traitement ou la prévention d’une hépatite virale.

12. Utilisation d’un quantité efficace au plan thérapeutique d’une souche d’IBDV vivante atténuée selon la revendication 1 ou la revendication 2 ou vecteur d’IBDV selon l’une quelconque des revendications 3 à 6 ou composition pharmaceutique selon l’une quelconque des revendications 7 à 8 pour la fabrication d’une composition pharmaceutique convenant à la prévention ou au traitement de l’hépatite virale.

13. Système comprenant (i) un vecteur d’IBDV déficient selon l’une quelconque des revendications 3 à 6 et (ii) une lignée cellulaire qui exprime la fonction manquante dudit vecteur.
14. Système selon la revendication 13, dans lequel l’édit vecteur d’IBDV est défficient en expression de polymérase d’ARN.
Fig. 1A - Segment A of IBDV V903/78 (SEQ. ID. NO: 1)
FIG. 1B - Segment B of IBDV V90378 (SEQ. ID. NO: 2)
Fig. 2
Fig. 3

Fig. 4
pA903Afl

A5'-903 → AflII → AflIII → NdeI → BsrGI

EcoRI

Transcription

pC903-INFa

A5'-903 sAa1 → sAa2 → AflIII → AflIII → NdeI → BsrGI

INF-alpha → IRES → VP2 → VP4 ↓ VP3

EcoRI → aAa2 → aAa3 → sAa2 → A3'-903

Transcription

Fig. 7
REFERENCES CITED IN THE DESCRIPTION

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